

STAT3, but not ERKs, mediates the IL-6-induced proliferation of renal cancer cells, ACHN and 769P

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STAT3, but not ERKs, mediates the IL-6-induced proliferation of renal cancer cells, ACHN and 769P.

Background. Although interleukin-6 (IL-6) has been suggested to function as an autocrine growth factor in renal cell carcinoma (RCC), the underlying mechanism responsible for the IL-6-induced proliferation of RCC has not been defined. The aim of this study was to characterize the signaling cascades mediating IL-6-induced proliferation and to investigate the use of effective novel interventions to block the IL-6-induced autocrine growth of renal cancer cells.

Methods. IL-6-induced proliferation and intracellular signaling cascades were analyzed in four human renal cancer cell lines Caki-1, ACHN, 769P and A498. IL-6-induced activation of STAT3 (signal transducer and activator of transcription-1) and extracellular signal-regulated kinases (ERKs), and the effects of anti-IL-6 neutralizing antibody, Jak inhibitor AG 490, and MEK1 inhibitor PD 98059 were analyzed by Western blotting using phospho-specific antibodies. The DNA-binding activities of STATs were analyzed by EMSA. Apoptosis was determined by using nuclear staining and the TUNEL assay. Changes in the apoptosis-related proteins, bcl-2, bcl-xL, and bax were analyzed by Western blotting.

Results. IL-6 induced tyrosine phosphorylation and increased the DNA binding activity of STAT3 and, to a lesser extent, STAT1 in all cell lines except for Caki-1, which did not express the IL-6 receptor subunit gp130. ERKs were constitutively activated in all cell lines and the activation level was not up-regulated further by exogenously added IL-6 nor down-regulated by anti-IL-6 neutralizing antibody. IL-6-induced STAT3 tyrosine phosphorylation and DNA binding activity was inhibited by treatment with Jak specific inhibitor AG 490; however, it was not affected by the MEK1 inhibitor PD 98059. Moreover, treatment with AG 490 inhibited IL-6-induced proliferation of ACHN and 769P cells and induced apoptosis with the down-regulation of bcl-2 and the up-regulation of bax.

Conclusions. This study identified STAT3, but not ERKs, to be a major mediator of IL-6-induced proliferation of renal cancer cells. Although ERKs were constitutively activated, ERKs were not found to be essential for the IL-6-induced proliferation and modulation of the STAT3 activity. Because the Jak

specific inhibitor AG 490 effectively inhibited the IL-6-induced STAT3 activity and induced apoptosis, the blockade of the STAT3 signaling pathways is considered to be potentially useful as a novel therapeutic approach for RCC.

Interleukin-6 (IL-6) is a cytokine with pleiotropic activities, including the induction of differentiation and the regulation of growth in a cell-type dependent manner [1]. IL-6 initiates its action by binding to its receptor, which is composed of two subunits: an 80-kD IL-6 binding protein (gp80) and a 130-kD transmembrane signal-transducing component (gp130) [2, 3]. The binding of IL-6 to its receptor induces the dimerization of gp130, the activation of the gp130-associated protein tyrosine kinases of the Janus kinase families (Jak1, Jak2 and Tyk2) and the phosphorylation of gp130 at cytoplasmic tyrosine residues [4]. These phosphotyrosines serve as docking sites for the SH2 domain containing proteins such as the signal transducers and activators of transcription (STAT1 and STAT3) and the protein tyrosine phosphatase (SHP2), which themselves subsequently become tyrosine phosphorylated [4, 5].

After tyrosine phosphorylation, STAT3 forms a homodimer or a heterodimer with STAT1 and thereafter enters the nucleus, where it regulates the expression of specific genes. In addition to tyrosine phosphorylation, STAT3 is serine phosphorylated at a single residue (Ser727) in response to IL-6 [6]. Although tyrosine phosphorylation has been shown to be essential for the dimerization, nuclear translocation and DNA binding of STAT3 [4], the functional significance of Ser 727 phosphorylation of STAT3 remains controversial [6–8].

On the other hand, SHP-2 has been shown to mediate IL-6-induced signals to the extracellular signal-regulated kinases (ERKs), which have been shown to play key roles in the intracellular signal transductions [5]. ERKs are activated in a variety of cell types by diverse extracellular stimulation and are among the most thoroughly studied of the signaling pathways that connect different membrane receptors to the nucleus [9].

In addition, recent studies have shown that STAT5

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can be activated by IL-6 independently of the tyrosine phosphorylation of gp130 [10], while the stress-activated protein kinase p38 is also activated by IL-6 [11]. Although STAT3 and ERKs have been reported to be major signaling pathways of IL-6, the molecules essential for the cell proliferation induced by IL-6 vary depending upon the cell type and the expression patterns of the signaling molecules [5, 12, 13]. Moreover, ERKs also have been reported to play some role in the modulation of STAT3 activity [7, 8, 14, 15].

Despite extensive evaluations of many different treatment modalities, metastatic renal cell carcinoma (RCC) remains highly resistant to systemic therapy [16]. As a result, the identification of new agents with better anti-tumor activity against metastases remains the highest priority in chemical studies of this refractory tumor. IL-6 has been suggested to function as an *in vitro* autocrine growth factor in RCC by demonstrating that primary RCC expressed IL-6, and that their *in vitro* growth was inhibited by an anti-IL-6 antibody [17, 18]. Patients with RCC often exhibit paraneoplastic syndrome, which includes fever, leukocytosis, erythrocytosis, hypercalcemia and an elevation of acute phase reactants. IL-6 has been reported to be involved in the physiopathology of the paraneoplastic syndromes [19]. Furthermore, the majority of patients with metastatic RCC demonstrate increased levels of serum IL-6 while patients with detectable serum IL-6 have poor survival [19]. These observations raise the possibility that pharmacological interventions to block IL-6-induced autocrine growth may have therapeutic potential in RCC. Unfortunately, the signaling mechanism involved in the IL-6-mediated growth of RCC remains to be elucidated.

In the present study, we characterized the signaling cascades mediating the IL-6-induced proliferation in renal cancer cells and investigated a novel pharmacological intervention to block IL-6-induced growth. Our results demonstrated that the response of renal cancer cells to IL-6 is most likely mediated by STAT3, but not ERKs. Moreover, ERKs were constitutively activated independently of IL-6 stimulation. In addition, ERK activity was not essential for modulation of STAT3 tyrosine phosphorylation and DNA-binding activity. We also demonstrated that Jak inhibitor AG 490 effectively inhibited the IL-6-induced activation of STAT3 without any effect on ERKs activity and induced apoptosis in renal cancer cells. Based on these findings, we postulate that a disruption of the IL-6-mediated signals to STAT3 is potentially useful as a novel therapeutic approach to RCC.

METHODS

Cell culture

Four established human renal cancer cell lines, Caki-1, ACHN, 769P, and A498 were obtained from ATCC (Rock-

ville, MD, USA). All cells were maintained in RPMI-1640 supplemented 10% fetal bovine serum (FBS), 50 IU/mL penicillin and 50 µg/mL streptomycin at 37°C in a humidified atmosphere with 5% CO₂.

Reagents

Human recombinant IL-6 was purchased from Peprotech (Rocky Hill, NJ, USA) and used at 20 ng/mL. Mouse monoclonal anti-human IL-6 neutralizing antibody (anti-IL-6 Ab) was obtained from R&D systems (Minneapolis, MN, USA). Jak inhibitor AG 490 and MEK1 inhibitor PD 98059 were from Calbiochem (La Jolla, CA, USA) and were solubilized in dimethylsulfoxide (DMSO) at 50 mmol/L, stored in the dark at -70°C. Phorbol 12-myristate 13-acetate (PMA), which is a strong stimulator of ERKs, was from Calbiochem. Rabbit polyclonal antibodies against STAT1, phospho-specific STAT1 at Tyr701 (p-STAT1), STAT3, phospho-specific STAT3 at Tyr705 (p-STAT3), phospho-specific STAT5 at Tyr694 (p-STAT5), p38, phospho-specific p38 at Thr180/Tyr182 (p-p38), ERKs, phospho-specific ERKs at Thr202/Tyr204 (p-ERKs) were from New England Biolabs (Beverly, MA, USA). Mouse monoclonal antibodies against STAT5, bcl-2, bcl-xL, bax, and mcl-1 were from BD Transduction Laboratories, Inc. (Franklin Lake, NJ, USA). Mouse monoclonal antibody against β-actin was from DAKO (Tokyo, Japan).

Determination of IL-6 secretion

The cells were plated at 1×10^4 cells/well in six-well plates in RPMI 1640 containing 10% FBS for 24 hours, and then were washed with serum-free medium and cultured for an additional 24 hours in 1 mL of the serum free medium. The medium was collected and clarified by centrifugation at $3000 \times g$. The levels of IL-6 in the supernatants were analyzed by enzyme-linked immunosorbent assay (ELISA) using the HS human IL-6 immunoassay kit (R&D Systems).

RNA extraction and RT-PCR analysis for IL-6 receptor gp80 and gp130 subunits

For RT-PCR, total RNA was isolated from 1×10^7 cells using Trizol according to the manufacturer's instructions (Gibco Life Technology, Rockville, MD, USA). One microgram RNA was reverse transcribed by Molony murine leukemia virus (MMLV) reverse transcriptase (Boehringer Mannheim, Mannheim, Germany) with hexamer random primer. For polymerase chain reaction (PCR), 2 µL cDNA was amplified using each gene specific primers in a total volume of 50 µL using 2 U Taq polymerase (Boehringer Mannheim). After 35 cycles, 15 µL aliquots were run on 1.5% agarose gels and stained with ethidium bromide. The nucleotide bases used were 5'-ACC TAT GAA GAT AGA CCA TCT AAA-3' as an upstream primer and 5'-GGT TCT ATA AAA TAT AGT ATA

ATT-3' as a downstream primer for gp130, 5'-ATG CTG GCC GTC GGC TGC GCG CTG-3' as an upstream primer and 5'-TCT GAG CTC AAA CCG TAG TCT-3' as a downstream primer for gp80, and 5'-TGA AGG TCG GAG TCA ACG GAT TTG GT-3' as an upstream primer and 5'-CAT GTG GGC CAT GAG GTC CAC CAC-3' as a downstream primer for GAPDH. The gp130, gp80 and GAPDH product sizes were 720 bp, 768 bp and 983 bp, respectively [20].

Western blotting

For analyses of intracellular signals by IL-6 in renal cancer cells, the cells were starved for 24 hours in serum-free RPMI-1640 and stimulated with IL-6 for the indicated time. The cells were put on ice and rinsed with ice-cold phosphate-buffered-saline (PBS) containing 100 $\mu\text{mol/L}$ Na_3VO_4 , and lysed with cell lysis buffer [20 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 2 mmol/L ethylenediaminetetraacetic acid (EDTA), 1% NP-40, 50 mmol/L NaF, 10 $\mu\text{g/mL}$ aprotinin, 10 $\mu\text{g/mL}$ leupeptin, 1 mmol/L phenylmethylsulfonyl fluoride (PMSF), 1 mmol/L Na_3VO_4]. The extracts were clarified by centrifugation at 12,000 r.p.m for five minutes. Lysates containing equal amounts of proteins were resolved by electrophoresis on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and then transferred to nitrocellulose membrane (Bio-Rad Laboratory, Hercules, CA, USA). The membranes were blocked with Tris-buffered saline (TBS) containing 5% nonfat milk before incubation with primary antibodies. Immunoreactive bands were visualized with the amplified alkaline phosphatase system according to the manufacturer's instructions (Bio-Rad). For analyzing the effect of AG 490 and PD 98059 on the intracellular signals, the cells were pretreated with serial dilutions of AG 490 (for 16 hours) or PD 98059 (for one hour) prior to stimulation with IL-6. For analyzing the changes in the expression levels of apoptosis-related proteins, the cells were incubated with IL-6 in the presence of 100 $\mu\text{mol/L}$ AG 490 for the indicated time. The cell lysates were prepared, and Western blotting was performed in the same manner.

Electrophoretic mobility shift assay (EMSA)

Nuclear extracts were prepared in hypertonic buffer [20 mmol/L HEPES pH 7.9, 420 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L egtazic acid (EGTA), 20% glycerol, 20 mmol/L NaF, 1 mmol/L Na_3VO_4 , 1 mmol/L $\text{Na}_4\text{P}_2\text{O}_7$, 1 mmol/L dithiothreitol (DTT), 0.5 mmol/L PMSF, 1 $\mu\text{g/mL}$ aprotinin, 1 $\mu\text{g/mL}$ leupeptin]. Twenty micrograms of the supernatants were incubated with 2 μg poly dI-dC (Amersham Pharmacia Biotech, Buckinghamshire, UK) with double-strand oligonucleotides for the consensus binding of sis-inducible-element (SIE; 5'-GTG CAT TTC CCG TAA ATC TTG TCT AGA-3') labeled with [γ - ^{32}P] dATP by T4 polynucleotide kinase (New England Bio-

labs) in binding buffer (10 mmol/L HEPES pH 8.0, 80 mmol/L NaCl, 1 mmol/L EDTA, 10% glycerol, 1 mmol/L DTT) for 20 minutes at room temperature. The samples were separated on a non-denaturing 4% polyacrylamide gel in 0.5% TBE. Thereafter, the gels were dried and autoradiographed.

Determination of cell viability

The effects of IL-6, anti-IL-6 Ab and AG 490 on cell viability of renal cancer cells were determined by using crystal violet staining as described previously [21]. In brief, the cells were plated into a flat-bottom 96-well plates at 1×10^4 cells/200 μL /well in triplicate in RPMI-1640 with 10% FBS. Twenty-four hours later, the medium was changed to serum-free medium containing the indicated reagents and incubated for the indicated time. After incubation, the plates were washed and cell viability was determined by staining the plates with 0.2% crystal violet (in 2% ethanol).

Nuclear staining

The cells were fixed with 4% paraformaldehyde for 30 minutes at room temperature and stained with 1 mmol/L Hoechst 33258 (Sigma, St. Louis, MO, USA) for 10 minutes at 37°C. The morphology was observed under a fluorescence microscope at a 365-nm-wavelength excitation light.

TUNEL analysis

DNA fragmentation was detected using the TUNEL technique as described previously [22]. In brief, 1×10^6 cells were fixed in 1% paraformaldehyde, permeabilized in 70% ethanol, and stored at -20°C . Breaks at 3'-OH DNA were detected by the TUNEL technique using the Apotag Plus Fluorescein (FITC) kit (Oncor, Gaithersburg, MD, USA) according to the supplier's instructions. FITC-labeled cells were counterstained with propidium iodide (PI; Sigma) and analyzed by flow cytometry using EPICS ALTRA flow cytometer (Beckman Coulter, Fullerton, CA, USA).

Quantification of the immunoreactive bands and statistical analysis

The relative density of immunoreactive bands of apoptosis-related proteins on Western blotting was calculated using the Image 1.52 program (NIH, Bethesda, MD, USA). The unpaired *t* test was used to determine statistical differences. A *P* value of less than 0.05 was considered to indicate statistical significance.

RESULTS

Production of IL-6 and expression of IL-6 receptor subunits in renal cancer cells

First, the production of IL-6 into the culture medium and the expression of IL-6 receptor subunits (gp80 and

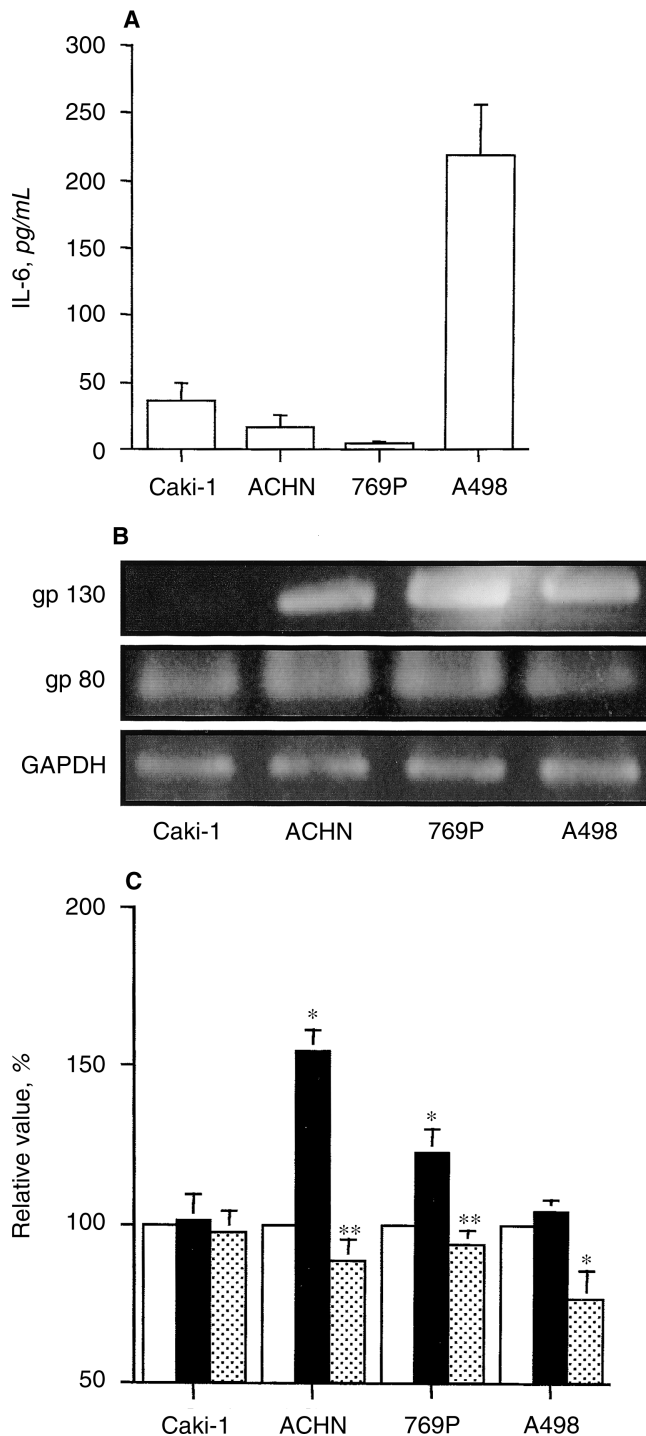


Fig. 1. (A) Determination of interleukin-6 (IL-6) production in the culture medium by renal cancer cells. The level of IL-6 production was determined using ELISA. Cells were seeded at equal densities (1×10^4 cells/well) in 6-well plates. Next the cells were cultured for 24 hours in serum free medium and the supernatants were analyzed with ELISA. The results represent the mean \pm SD of three separate experiments. (B) RT-PCR analysis for the expression of the IL-6 receptor (gp80) and gp130 mRNA in renal cancer cells. The gp130, gp80 and GAPDH product sizes were 720 bp, 768 bp and 983 bp, respectively. GAPDH was used as an internal control. (C) Cell proliferation in response to IL-6. Cells were incubated in the presence or absence of 20 ng/mL IL-6 with or without 1.5 μ g/mL anti-human IL-6 antibody (Ab) for 96 hours. Symbols are: (□) untreated; (■) IL-6; (▨) IL-6 + anti-IL-6 Ab. Cell

gp130) were confirmed in renal cancer cells. Basal IL-6 secretion levels were determined using ELISA. Although all cell lines exhibited detectable basal production of IL-6 (35.3 ± 13.8 pg/mL in Caki-1, 16.3 ± 9.3 pg/mL in ACHN, 4.2 ± 2.0 pg/mL in 769P, and 219 ± 37 pg/mL in A498, respectively), the levels of IL-6 production varied widely depending upon the cell type (Fig. 1A). Expression of mRNA for gp80 and gp130 was determined by the RT-PCR methods. The specific product for gp80 subunit (768 bp) was observed in all cell lines, whereas gp130 (720 bp) was expressed in ACHN, 769P and A498, but not in Caki-1 (Fig. 1B).

Effect of exogenously added IL-6 and anti-IL-6 Ab on the proliferation of renal cancer cells

The effects of exogenously added IL-6 and anti-IL-6 Ab on the proliferation of renal cancer cells were assessed by crystal violet staining. According to the manufacturer's specifications, 1.5 μ g/mL of the anti-IL-6 Ab almost completely neutralized the biological activity due to 20 ng/mL IL-6. Incubation with IL-6 increased ACHN and 769P cells growth by $154.6 \pm 6.7\%$ and $122.8 \pm 7.1\%$, respectively, after 96 hours in comparison to the untreated cells ($P < 0.05$; Fig. 1C). Moreover, preincubation with anti-IL6 Ab significantly inhibited IL-6-induced cell proliferation of ACHN and 769P cells ($P < 0.05$; Fig. 1C). Although no significant proliferation by exogenously added IL-6 was observed in A498 cells, anti-IL-6 Ab significantly inhibited proliferation compared with untreated cells ($P < 0.05$; Fig. 1C). In Caki-1, which did not express gp130 mRNA, IL-6 and anti-IL-6 Ab had no effect on the proliferation (Fig. 1C).

Analyses of intracellular signals of IL-6 in renal cancer cells

The intracellular signaling pathways of IL-6 in renal cancer cells were analyzed by Western blotting using phospho-specific antibodies, which only detect the activated status. First, the effect of IL-6 on the tyrosine phosphorylation levels of STAT1, STAT3 and STAT5 (STAT1 at Tyr701, STAT3 at Tyr705, and STAT5 at Tyr694, respectively) was examined. To assess the phosphorylation levels of STAT proteins, starved cells were either left unstimulated or were stimulated with IL-6 for 15 minutes. Total cell extracts were prepared and subjected to Western blotting. Total STAT1, STAT3, and STAT5 proteins were present in all cell lines and the expression levels were not affected by IL-6 (Fig. 2A). IL-6 induced comparable tyrosine phosphorylation of

viability was determined by crystal violet staining. Results represent the mean \pm SD of three separate experiments. * $P < 0.05$ compared with untreated cells. ** $P < 0.05$ compared with the cells treated with IL-6.

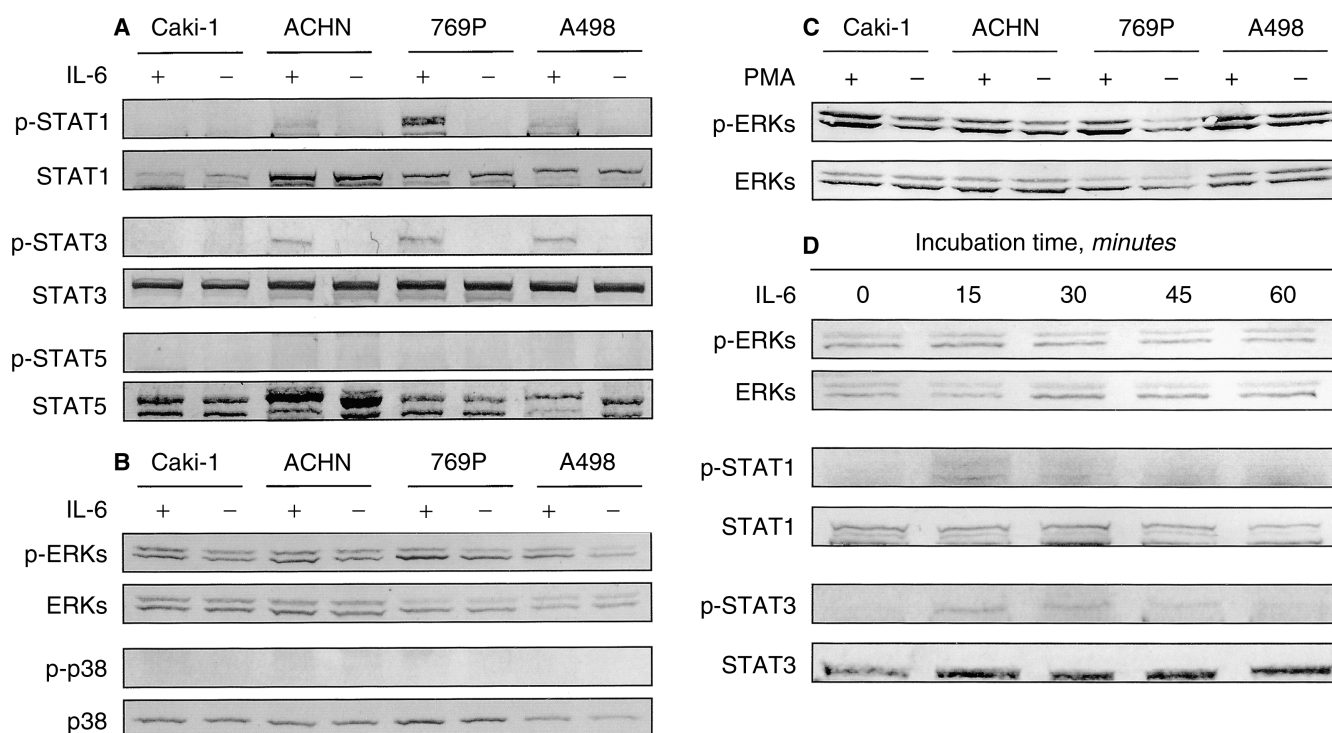


Fig. 2. IL-6-mediated intracellular signaling cascades in renal cancer cells. Serum deprived cells were stimulated with 20 ng/mL IL-6 for 15 minutes. The phosphorylation levels of STAT1, STAT3, STAT5 (A), ERKs and p38 (B) were analyzed by western blotting using phospho-specific antibodies, which detect only activated status. (C) Serum deprived cells were untreated or treated with 100 ng/mL PMA for 10 minutes and the phosphorylation level of ERKs was analyzed by Western blotting (D). The phosphorylation kinetics of STAT1, STAT3 and ERKs in ACHN cells after stimulation with IL-6. Serum deprived cells were stimulated with IL-6 for 15 to 60 minutes and phosphorylation level was analyzed by Western blotting.

STAT3 and, to a lesser extent, STAT1 in all cell lines except for Caki-1, which did not express gp130 (Fig. 2A). The amount of phosphorylated STAT1 seemed to be far less than that of phosphorylated STAT3 because the exposure time to detection reagents required to visualize the immunoreactive bands of phosphorylated STAT1 is much longer than phosphorylated STAT3. IL-6 did not induce tyrosine phosphorylation of STAT5 in all cell lines (Fig. 2A).

To determine whether IL-6 activated the ERKs and p38 cascades, their activation status was assessed by Western blotting using antibodies that specifically recognize the dually phosphorylated status (ERKs at Thr202/Tyr204 and p38 at Thr180/Tyr182, respectively). In all cell lines, total ERKs and p38 were expressed at comparable levels, which were not affected by IL-6 (Fig. 2B). Interestingly, ERKs were constitutively activated in all cell lines, which were not further up-regulated by stimulation with exogenous IL-6 (Fig. 2B). A strong stimulator for ERKs phosphorylation, PMA, increased ERK phosphorylation in Caki-1 and 769P cells, which strongly support our results, indicating that IL-6 did not up-regulate ERK phosphorylation (Fig. 2C). On the other hand, p38 was not activated by IL-6 in any of the cell lines (Fig. 2B).

Furthermore, to study the phosphorylation kinetics of

STAT1, STAT3 and ERKs in response to IL-6, ACHN cells were stimulated with IL-6 for 15 to 60 minutes and the phosphorylation levels were analyzed by Western blotting. The transient tyrosine phosphorylation of STAT3 and, to a lesser extent, STAT1 was observed within 15 minutes upon IL-6 stimulation in ACHN cells and returned to near basal level within approximately 60 minutes, whereas the phosphorylation level of ERKs showed no changes from the unstimulated level at any periods (Fig. 2D). The phosphorylation of STAT5 and p38 was not observed at any period (data not shown). The same results also were obtained for 769P cells (data not shown).

Endogenous IL-6 produced by renal cancer cells was not responsible for the constitutive activation of ERKs

Because ERKs have been shown to be a major mediator of IL-6 signaling [5, 12] and renal cancer cells secreted detectable levels of IL-6 into a culture medium (Fig. 1A), we questioned whether endogenous IL-6 produced by renal cancer cells might be responsible for the constitutive activation of ERKs. We examined whether the neutralization of endogenous IL-6 activity might lead to a decrease in the phosphorylation levels of ERKs. The starved ACHN and 769P cells were untreated, stimulated with IL-6 for 15 minutes, cultured for 24 hours with anti-

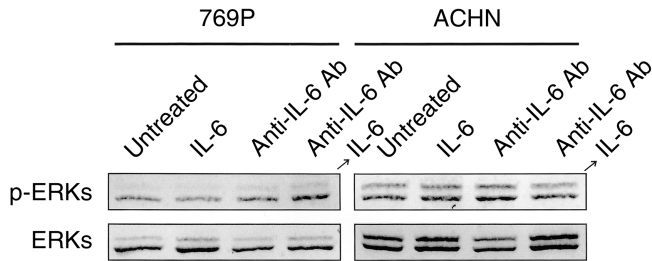


Fig. 3. Neutralization of IL-6 did not reduce the phosphorylation levels of extracellular signal-regulated kinases (ERKs). Serum deprived 769P and ACHN cells were untreated, stimulated with IL-6 for 15 minutes, cultured with anti-IL-6 Ab for 24 hours, or cultured with anti-IL-6 Ab for 24 hours prior to stimulation with IL-6 for 15 minutes. Cell lysates were prepared and analyzed by western blotting.

IL-6 Ab, or cultured for 24 hours with anti-IL-6 Ab prior to stimulation with IL-6, and then the phosphorylation levels of ERKs were analyzed by Western blotting. In both cell lines, the levels of ERKs phosphorylation were not down-regulated by treating the cells with anti-IL-6 Ab nor up-regulated by any subsequent stimulation with exogenous IL-6 (Fig. 3). The same results also were seen in Caki-1 and A498 cells (data not shown). These results indicate that constitutive ERK phosphorylation does not appear to be due to the endogenous IL-6 produced by renal cancer cells.

The Jak-specific inhibitor AG 490 attenuated IL-6-induced tyrosine phosphorylation of STAT3, but not constitutive phosphorylation of ERKs

Interleukin-6 leads to the homodimerization of gp130, which results in the activation of gp130-associated Jak1, Jak2 and Tyk2. Subsequently, gp130 is phosphorylated on tyrosine, and the phosphorylated molecule recruits SHP2 and STAT3 [4, 5]. To investigate the involvement of Jak kinase in IL-6-mediated signaling cascades in renal cancer cells, we examined whether treatment with the Jak-specific inhibitor AG 490 attenuates the phosphorylation levels of STAT3 and ERKs. ACHN and 769P cells were exposed to various concentrations of AG 490 16 hours prior to stimulation with IL-6. Preincubation with AG 490 resulted in the inhibition of the IL-6-induced tyrosine phosphorylation of STAT3 at 25 $\mu\text{mol/L}$ in ACHN cells and at 100 $\mu\text{mol/L}$ in 769P cells (upper panels, Fig. 4). On the other hand, the constitutive phosphorylation of ERKs was not suppressed by AG 490, even at high concentrations (100 $\mu\text{mol/L}$) in both cell lines (lower panels, Fig. 4). These results indicate that Jak kinase is essential for the IL-6-induced activation of STAT3, but not for the constitutive activation of ERKs in renal cancer cells.

Inhibition of constitutively activated ERKs by the MEK1 inhibitor PD 98059 did not modulate the IL-6-induced tyrosine phosphorylation of STAT3

The functional significance of ERKs in the modulation of the STAT3 activity has been reported to vary ac-

cording to the cell type and activation status [7, 8, 14, 15]. ERKs have been reported to potentiate the STAT3 activity through serine phosphorylation [8], while inhibiting the STAT3 activity upstream from tyrosine phosphorylation [15]. Since the ERKs were constitutively activated in all cell lines in our experiments, we speculated that ERKs was involved to some degree in the modulation of STAT3 activity. To examine whether the inhibition of the ERK activity by PD 98059, which is a specific inhibitor of MEK1, alter the level of IL-6-induced tyrosine phosphorylation of STAT3 in renal cancer cells, ACHN and 769P cells were exposed to various concentrations of PD 98059 one hour prior to stimulation with IL-6. PD 98059 inhibited the constitutively activated ERKs at 25 $\mu\text{mol/L}$ in both cell lines, whereas it did not affect the tyrosine phosphorylation levels of STAT3 induced by IL-6 even at 100 $\mu\text{mol/L}$ (Fig. 5). These results indicate that the ERK activity is not essential for the modulation of STAT3 activation at the tyrosine phosphorylation level.

IL-6-induced STATs-DNA binding activity is inhibited by AG 490, but not by PD98059

In addition to tyrosine phosphorylation, we examined whether IL-6 increases the STATs-DNA binding activity and whether AG 490 or PD 98059 suppresses the DNA binding activity by EMSA using a ^{32}P -labeled SIE probe, which binds both activated STAT1 and STAT3 with a high affinity [23, 24]. The stimulation of ACHN and 769P cells with IL-6 resulted in the rapid induction of a STATs-DNA complex that binds the SIE oligonucleotides (Fig. 6). The IL-6-induced STATs-DNA binding activity was markedly inhibited by AG 490, but not PD 98059, and this finding closely correlated with that of the tyrosine phosphorylation level (Fig. 6). These results indicate that the ERK activity is not involved in the modulation of the STAT3 activity at the nuclear translocation level.

AG 490 suppressed the IL-6-induced proliferation of renal cancer cells

Previous results indicated that IL-6 mainly activates STAT3, while Jak inhibitor AG 490 effectively inhibits the IL-6-induced STAT3 tyrosine phosphorylation and DNA binding activity in ACHN and 769P cells. To study the functional consequences following the inhibition of IL-6 signaling by Jak inhibitor, the effect of AG 490 on IL-6-induced proliferation of renal cancer cells was tested on ACHN and 769P cells that had been treated with IL-6 in the presence or absence of AG 490 at serial concentrations for 96 hours. The proliferation of both cell lines was significantly suppressed following AG 490 treatment in a dose dependent manner (Fig. 7). The proliferation of ACHN cells was significantly suppressed by AG 490 at a concentration ≥ 1 $\mu\text{mol/L}$, with $\text{IC}_{50} = 0.5$ $\mu\text{mol/L}$

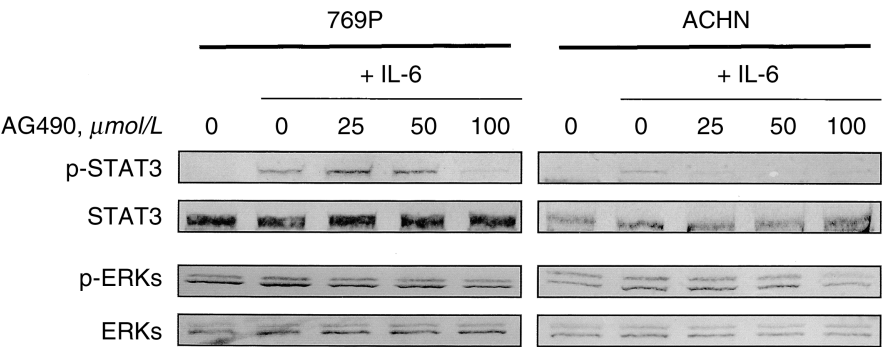


Fig. 4. Effect of the Jak inhibitor AG 490 on the phosphorylation of STAT3 and ERKs in IL-6-stimulated renal cancer cells (RCC). Serum deprived 769P and ACHN cells were untreated, stimulated with IL-6 for 15 minutes, or treated with serial concentration of AG 490 for 16 hours prior to stimulation with IL-6 for 15 minutes. Cell lysates were prepared and subjected to Western blotting.

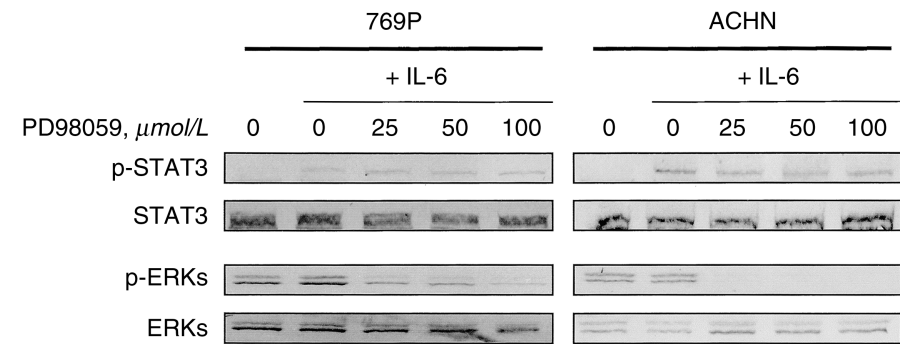


Fig. 5. Effect of MEK1 inhibitor PD 98059 on the phosphorylation of STAT3 and ERKs in IL-6-stimulated renal cancer cells. Serum-deprived 769P and ACHN cells were untreated, stimulated with IL-6 for 15 minutes, or treated with serial concentration of PD 98059 for 1 hour prior to stimulation with IL-6 for 15 minutes. Cell lysates were prepared and subjected to Western blotting.

and 769P cells also was suppressed at a concentration $\geq 1.0 \mu\text{mol/L}$, with an $\text{IC}_{50} = 3.5 \mu\text{mol/L}$. The toxic effects from the solvent used to dissolve AG 490 could be excluded since the cells incubated with the highest concentration of DMSO, which was equivalent to $100 \mu\text{mol/L}$ AG 490, showed no significant growth inhibition (data not shown). To examine any other effects of AG 490, the effects of AG 490 on the proliferation of 769P and ACHN cells were examined without IL-6 stimulation, which resulted in significant growth suppression (data not shown). However, under non-stimulated conditions, the concentration of AG 490 required to significantly suppress the spontaneous growth was higher than IL-6-stimulated condition ($\text{IC}_{50} = 10.0 \mu\text{mol/L}$ in both cells). In addition, AG 490 suppressed proliferation of Caki-1 cells, which had no proliferative response to IL-6 (data not shown). However, the concentration of AG 490 required to suppress proliferation of Caki-1 cells also is much higher than to suppress the IL-6-driven proliferation of 769P and ACHN cells (IC_{50} value = $30.0 \mu\text{mol/L}$ in Caki-1 cells). Based on these results, the spontaneous and IL-6-induced growth of renal cancer cells seems to be mediated by at least partly distinct pathways with different sensitivities to AG 490. Similar results indicating different sensitivities to AG 490 between cytokine-driven growth and spontaneous growth have been reported in another study [25].

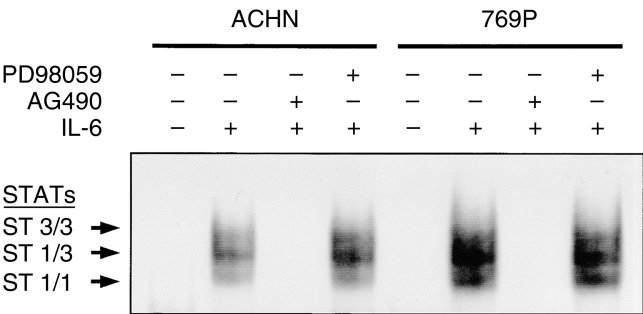


Fig. 6. AG 490, but not PD 98059 inhibited increased STATs-DNA binding activity by IL-6 in ACHN and 769P cells. Serum-deprived 769P and ACHN cells were untreated, stimulated with IL-6, or incubated with $100 \mu\text{mol/L}$ AG 490 for 16 hours or $100 \mu\text{mol/L}$ PD 98059 for one hour prior to stimulation with IL-6 for 15 minutes. Nuclear extracts were incubated with the ^{32}P -labeled SIE oligonucleotide probe, which binds both activated STAT1 and STAT3, and then were analyzed by EMSA. The positions of STAT3 homodimer (ST3/3), STAT1/STAT3 heterodimer (ST1/3), STAT1 homodimer (ST1/1), are indicated on the left.

AG 490 induced apoptosis in renal cancer cells

Activated STAT3 signaling has been shown to prevent apoptosis [13, 26, 27], and to determine whether the inhibition of IL-6-induced signals by AG 490 may lead to apoptotic cell death, nuclear staining was done using a Hoechst 33258 and a flow cytometric TUNEL assay, both of which are useful methods for detecting apoptosis. ACHN cells were either untreated or incubated with IL-6

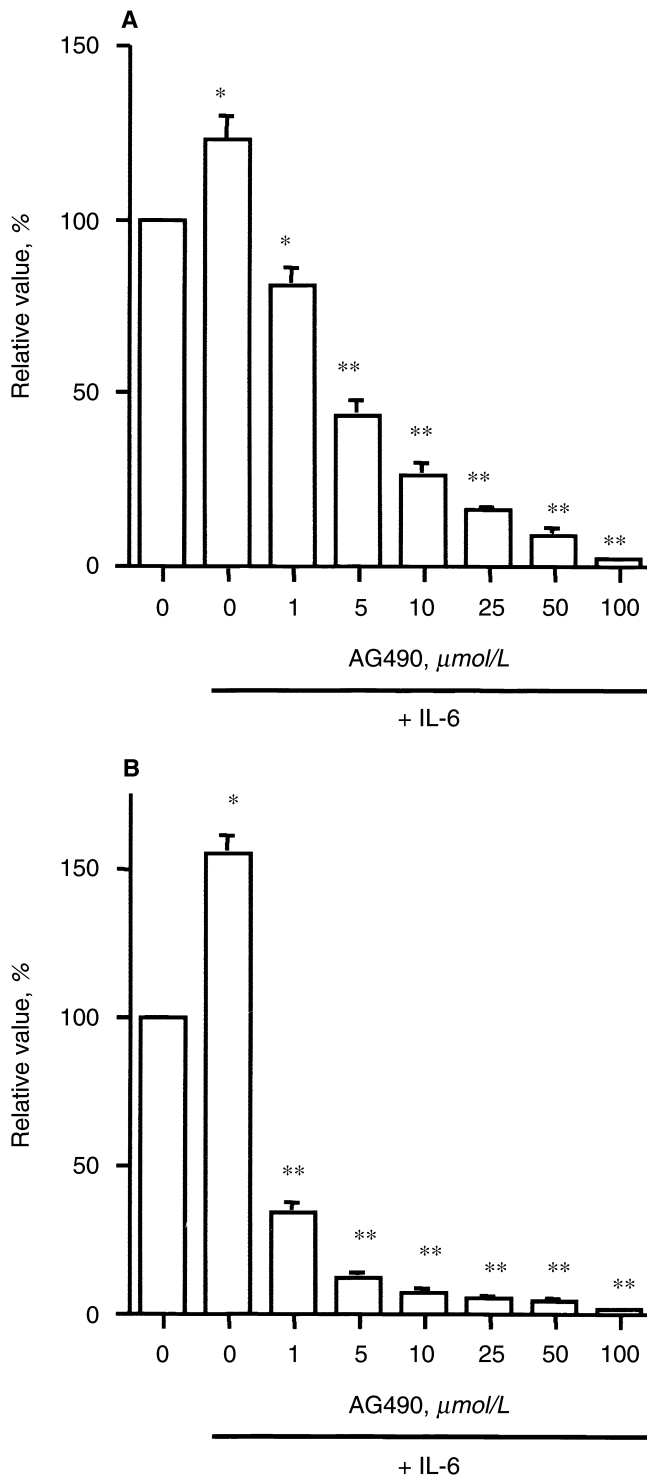


Fig. 7. Effects of AG 490 on the proliferation of renal cancer cells in response to IL-6. ACHN (A) and 769P (B) cells were plated into a flat-bottom 96-well plates at 1×10^4 cells/200 μL /well in RPMI-1640 with 10% FBS. Twenty-four hours later, medium was changed to serum-free medium with or without IL-6 in the presence or absence of the indicated concentrations of AG 490 and incubated for 96 hours. The cell viabilities were determined by using crystal violet staining. The results are shown as the mean \pm SD of triplicate cultures. * $P < 0.05$ compared with untreated cells. ** $P < 0.01$ compared with untreated cells.

in the absence or the presence of 100 $\mu\text{mol/L}$ AG 490 for 96 hours and then stained with the DNA-specific fluorescent dye Hoechst 33258 for morphological examination. The nuclei of the cells treated with IL-6 in the presence of AG 490 appeared lobated and severely fragmented and contained highly condensed chromatin (Fig. 8A), which are characteristic findings of apoptosis. Furthermore, AG 490-induced apoptosis also was confirmed by a flow cytometric TUNEL assay. The untreated cells and cells cultured with IL-6 in the absence of AG490 showed apoptosis in only 0.8% and 0.4% of cells, respectively, whereas the cells cultured with IL-6 in the presence of 100 $\mu\text{mol/L}$ AG 490 showed apoptosis in 34.9% of cells (Fig. 8B). These apoptosis-inducing effects were observed in 769P cells as well (data not shown).

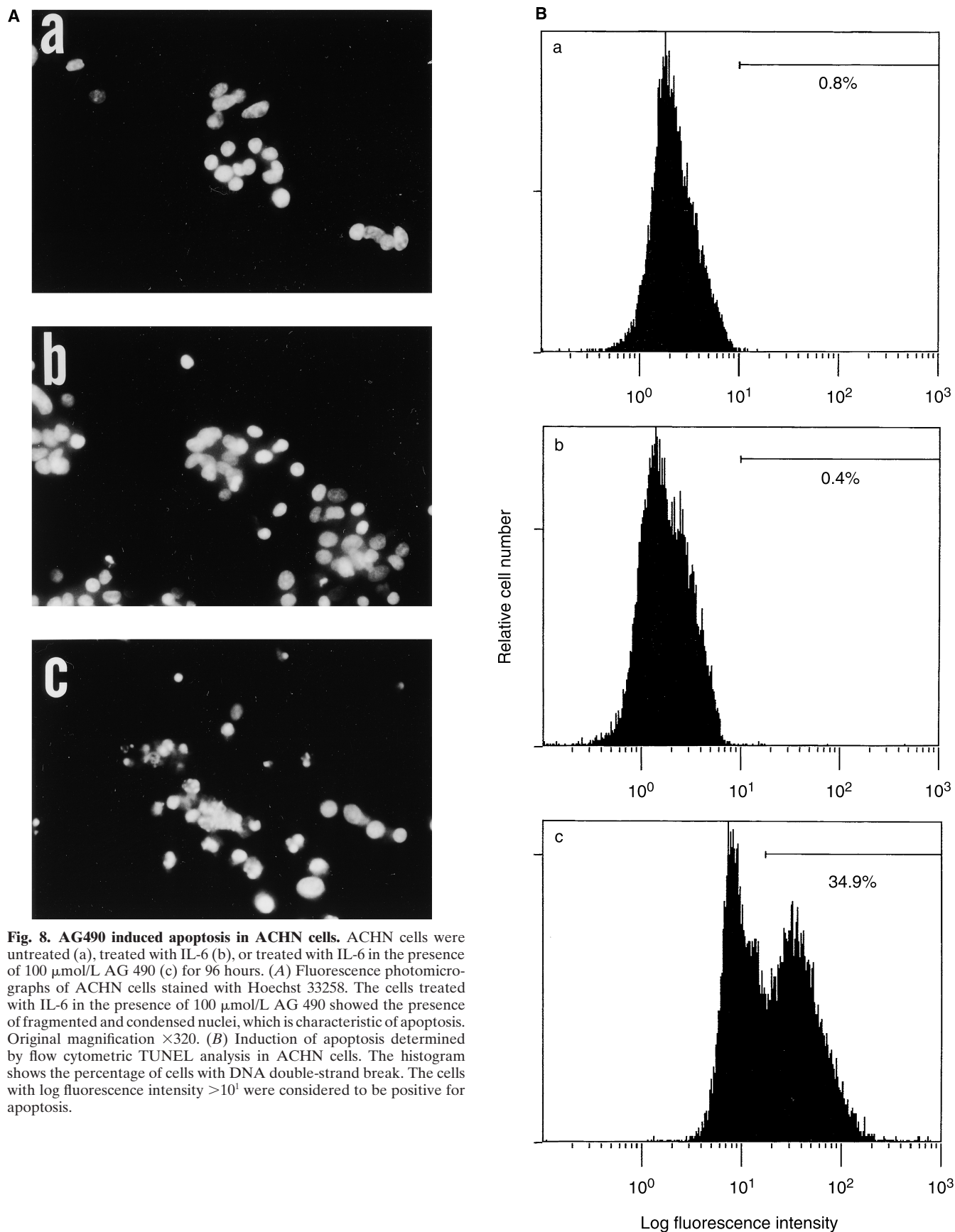
Changes in apoptosis-related proteins levels in AG 490 induced apoptosis

To further clarify the mechanisms underlying the AG 490-induced apoptosis of renal cancer cells, we examined whether AG 490 induces apoptosis by modulating the expression of apoptosis-related proteins, bcl-2, bcl-xL, mcl-1, and bax by Western blotting. ACHN cells were incubated with IL-6 in the presence of 100 $\mu\text{mol/L}$ AG 490 for the indicated times and thereafter were subjected to Western blotting. Although the bcl-2 and bax proteins were expressed at basal levels in ACHN, the expression of bcl-xL protein was very modest (Fig. 9) and mcl-1 could not be detected (data not shown). The inhibition of the Jak kinase activity by AG 490 resulted in the down-regulation of bcl-2 by 66.9% and the up-regulation of bax by 153.5% following 96 hours of treatment compared with the basal levels (Fig. 9). On the other hand, the expression of bcl-xL did not change (Fig. 9).

DISCUSSION

Although IL-6 has been shown to play an important role in the proliferation of RCC [17–19], the molecular basis for IL-6-mediated signaling cascades has not been previously investigated. To the best of our knowledge, this is the first report that characterized the signaling cascades mediating the IL-6-induced proliferation of renal cancer cells.

The present study first confirms the role of IL-6 in the proliferation of renal cancer cells. In our experiments, a significant IL-6-induced proliferation was observed in ACHN and 769P cells. Although A498 cells produced IL-6 at higher levels than other cells and expressed both IL-6 receptor subunits, no significant IL-6-induced proliferation was observed at 20.0 ng/mL. The result that the anti-IL-6 antibody suppressed proliferation of A498 cells under non-stimulated as well as IL-6 stimulated condition suggests that the proliferation of A498 cells depends at least in part upon IL-6. In our additional experi-



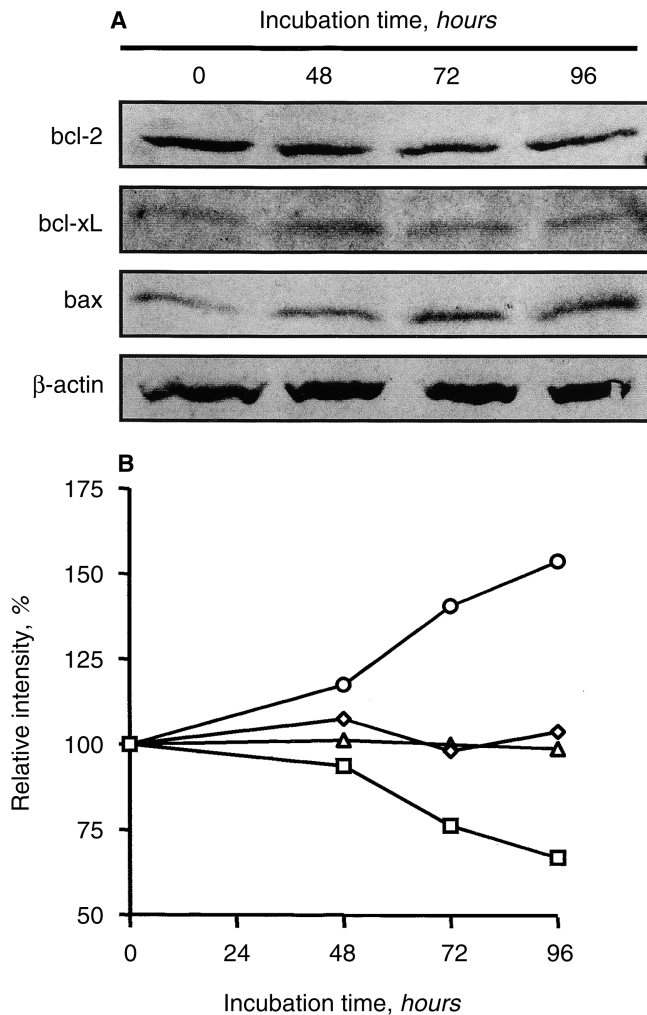


Fig. 9. (A) Western blot analysis for apoptosis-associated proteins bcl-2, bcl-xL, and bax. ACHN cells were cultured with IL-6 in the presence of 100 μ mol/L AG 490 for the indicated periods. Whole cell lysates were prepared and subjected to western blotting. β -actin was used as an internal control. (B) The relative intensity of the immunoreactive bands of (□) bcl-2; (◇) bcl-xL; (○) bax; (△) β -actin in comparison to the basal levels.

ments, the proliferation of A498 cells was significantly stimulated by exogenous IL-6 at a tenfold higher concentration (200.0 ng/mL). According to various reports, IL-6 exerts its biological activity in the concentration range of 0.1 ng/mL to 100.0 ng/mL, which is considered to be a physiological range [12, 15, 20, 28]. In fact, the serum IL-6 levels in patients with RCC have been reported between 1 pg/mL to 1850 pg/mL [19]. As a result, the exogenous IL-6-driven proliferation of A498 cells seems to be observed only under non-physiological conditions. A possible explanation for IL-6-induced STAT3 activation without a proliferative response is that activation level of STAT3 might be insufficient for the IL-6-driven proliferation up to 20 ng/mL or that IL-6-induced activation of STAT3 might be independent of the growth stimulation

in A498 cells up to 20 ng/mL. The lack of gp130 mRNA expression, which is essential for IL-6-mediated signaling [2, 3], could account for the unresponsiveness of Caki-1 to IL-6 and anti-IL-6 Ab. From these results, we confirmed that IL-6 acts as an autocrine or paracrine growth factor in renal cancer cells and these findings closely correlate with those of previous studies [17, 18].

To investigate which molecules were involved in the IL-6-mediated proliferation of renal cancer cells, we took into consideration that IL-6 acts as a growth factor for a variety of malignant tumors, in addition to RCC [12, 13, 20, 29]. Although STAT3 and ERKs have been reported to be major signaling pathways of IL-6 and recent studies showed that STAT5 and stress-activated protein kinase p38 are also activated by IL-6, the cascades mediating IL-6-induced growth vary depending upon the cell type. For example, in a B9 murine IL-6-dependent hybridoma cell line and some human multiple myeloma cells, the activation of STAT3 occurs independently of the proliferative response to IL-6, and their growth depends upon the activation of ERKs [12]. On the other hand, IL-6 stimulates prostate cancer cell growth accompanied by the activation of the STAT3 signaling pathway [29]. Moreover, STAT3 signaling is essential for the survival of IL-6-dependent human myeloma cell line, U266 cells [13]. In our experiments, IL-6 induced tyrosine phosphorylation and DNA binding activity of STAT3 and, to a lesser extent, STAT1 in all cell lines except for Caki-1 without any expression of gp130. We cannot completely exclude the possibility that STAT1 might contribute to IL-6-driven proliferation. However, recent studies revealed that STAT1 actually has tumor suppressor functions and loss of STAT1 signaling enhances oncogenesis [30–32]. On the other hand, STAT3 is believed to play an important role in the unrestrained growth of many human tumors. This may be a plausible explanation for our results in which the growth inhibitory effects of activated STAT1 can be overcome by the simultaneous activation of STAT3, which resulted in cell proliferation. The activation of STAT3 was attenuated by treatment with Jak inhibitor AG490 in ACHN and 769P cells, which proliferated by IL-6. The IL-6-induced proliferation of ACHN and 769P cells was significantly inhibited by AG 490. Although STAT5 and p38 were expressed in all cells, their activation was not observed after stimulation with IL-6. On the other hand, ERKs were constitutively activated in all cell lines, even in Caki-1 cells, which did not express gp130, and the activation level of ERKs was not further up-regulated by exogenously added IL-6 nor down-regulated by anti-IL-6 Ab. Furthermore, the inhibition of IL-6-mediated signal at the Jak kinase level by AG 490 did not decrease the degree of ERK phosphorylation. Based on these findings, we conclude that STAT3, but not ERKs, mediates the IL-6-induced proliferation of renal cancer cells.

The exact mechanism underlying the constitutive activation of ERKs in RCC is not known at present. The constitutive activation of ERKs has been shown to occur in the majority of RCC and the levels of activated ERKs increased with increasing tumor grade and stage [33]. ERKs are activated by a variety of mitogens such as epidermal growth factor (EGF) and transforming growth factor (TGF)- α [34]. In addition to IL-6, EGF and TGF- α stimulate the growth of renal cancer cell lines in an autocrine manner [35]. We speculate that such growth factors produced by renal cancer cells may be, at least in part, responsible for the constitutive activation of ERKs.

Studies have revealed that ERKs were involved in the modulation of STAT3 activity [7, 8, 14, 15]. ERKs were reported to potentiate STAT3 activity through serine phosphorylation [8], as well as to inhibit the STAT3 activity upstream of tyrosine phosphorylation [15]. The relative intensity of ERKs activity determines the modulation of the STAT3 activity [7]. Although ERKs were constitutively activated independently of IL-6, we speculated ERKs plays some role in the modulation of the STAT3 activity. We therefore investigated the role of ERKs in relation to the modulation of STAT3 activity at the level of tyrosine phosphorylation and DNA binding activity by using the MEK1 inhibitor PD 98059. Since the inhibition of the ERK activity by PD 98059 did not modulate the tyrosine phosphorylation or the DNA binding activity of STAT3 in renal cancer cells, we conclude that the ERK activity is not essential for the modulation of the IL-6-induced STAT3 activation.

The ERK and STAT3 signaling pathways have been shown to be essential for IL-6-mediated cell proliferation in other cells [5]. The ERK activation was shown to be essential for mitogenic signals, while, on the other hand, the STAT3 activation has been shown to be involved in the anti-apoptotic signals [5]. Accumulating evidence suggests that the STAT3-mediated inhibition of apoptosis may be caused by the regulation of apoptotic regulatory proteins, such as anti-apoptotic protein bcl-2 and bcl-xL [27]. Moreover, STAT3 regulates the transcription from the bcl-x promoter [13], and cells transformed by constitutively activated STAT3 also have elevated levels of bcl-xL mRNA [26]. Because we demonstrated that STAT3, but not ERKs, mediated the IL-6-induced proliferation of renal cancer cells, we speculated IL-6 might induce cell proliferation by preventing apoptosis via the STAT3 signaling pathway.

AG 490, a newly identified Jak inhibitor, was first shown to inhibit active DNA synthesis and it blocks the growth of recurrent pre-B-cell acute lymphoblastic leukemia cells in which Jak2 is constitutively activated both in vitro and in vivo by inducing apoptosis [36]. The efficacy of AG 490 in vivo is accompanied by a complete absence of toxicity to normal blood cells [36]. It has been demonstrated that AG 490 is not a general kinase inhibitor

because of its absence of any effects on other tyrosine kinases such as Lck, Lyn, Btk, Syk, or Src [36]. Recent studies revealed that AG 490 inhibit Jak1 and Jak3, in addition to Jak2, all of which are closely related members of the Jak kinase families [37, 38]. However, no other additional AG 490-inhibited kinases have been reported and AG 490 does not inhibit growth of Jak-negative Jurkat cells and Bcr-Abl fusion protein positive K562 cells, and these findings strongly support the specificity of AG 490 to Jak kinase [37, 39]. We cannot completely exclude the possibility that AG 490 inhibits signaling cascades other than STAT3. Because AG 490 is likely to be a Jak specific inhibitor, it may inhibit any other several downstream molecules that are associated with Jak kinase. In addition to STAT3, to date several signaling pathways are inhibited by AG 490, such as STAT5 and ERKs [37, 38]. However, we showed that STAT5 is not activated in either basal or IL-6-stimulated conditions and that constitutively activated ERKs were not inhibited by AG 490 even at 100 μ mol/L. Thus, the possibility that AG 490 might inhibit additional signals, at least for STAT5 and ERKs, can be excluded. In addition, the effects of AG 490 on the spontaneous growth of renal cancer cells resulted in significant growth suppression. However, the concentration required to suppress the spontaneous growth was much higher than that required to suppress the IL-6-induced growth. Similar results indicating different sensitivities to AG 490 between cytokine-driven growth and spontaneous growth were reported in another study [25]. We speculate that the difference between the concentrations of AG 490 required to suppress spontaneous growth and IL-6-driven growth is due to the difference between the basal and IL-6-induced Jak kinase activity. Based on these findings, we believe that AG 490-induced apoptosis is mediated, at least in part, by an inhibition of STAT3 activity.

Recent studies showed that AG 490 has been shown to block activation of STAT3 and growth of various cancer cells by the induction of apoptosis [13, 25, 29]. Therefore, we investigated whether the inhibition of IL-6-induced STAT3 activation by the Jak inhibitor AG 490 subsequently leads to an inhibition of cell proliferation by induction of apoptosis with a modulation of anti-apoptotic proteins bcl-2, bcl-XL, mcl-1 and the pro-apoptotic protein bax. In line with previous reports, AG 490 inhibited both the IL-6-induced STAT3 activation and IL-6-triggered proliferation of ACHN and 769P cells by the induction of apoptosis with a down-regulation of bcl-2 and up-regulation of bax. Whereas the changes in their expressions by AG 490 were very modest, other apoptosis-related proteins may be involved. The anti-apoptotic proteins bcl-2, bcl-xL, and mcl-1 have all been shown to be capable of binding to pro-apoptotic protein bax and suppressing bax-induced apoptosis [40]. Among the bcl-2 family proteins, bcl-2 is frequently expressed

in RCC, and a bcl-2 overexpression may explain the relative resistance of RCC to conventional chemotherapy and radiotherapy [41, 42]. Unfortunately, the regulation of the expression of bcl-2 family proteins in RCC is still poorly understood. Because Jak inhibitor AG 490 inhibited the IL-6-induced STAT3 activation followed by a reduction in bcl-2 expression, we speculate that the expression of bcl-2 may be regulated, at least in part, by STAT3 in RCC.

A surgical resection of the primary localized RCC remains the mainstay of therapy and metastatic RCC is highly refractory to conventional therapies including radiation and chemotherapy [16]. As a result, the management of advanced RCC remains a significant challenge to clinicians. Our results predict that blocking the IL-6-induced signaling by inhibiting STAT3 activity will not only block tumor growth by inducing apoptosis, but also may increase the sensitivity of tumors to conventional chemotherapy and radiation therapy by a reduction of the anti-apoptotic proteins, such as bcl-2.

In summary, we identified STAT3, but not ERKs, to be a major mediator of IL-6-induced proliferation of renal cancer cells. Although ERKs were constitutively activated, ERKs were not essential for the IL-6-induced proliferation and modulation of STAT3 activity. Because the Jak-specific inhibitor AG 490 effectively inhibited IL-6-induced STAT3 activity and induced apoptosis, the blockade of the STAT3 signaling pathway may be an effective and novel therapeutic approach for RCC.

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